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Dedicated to Professor Ionel Haiduc
on the occasion of his 65th birthday

DETERMINATION OF SOME AMINOACIDS FROM PHARMACEUTICAL PRODUCTS BY USING A KINETIC METHOD BASED ON A CLOCK LANDOLT-TYPE SYSTEM OF REDOX REACTIONS

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ABSTRACT. A kinetic method is described for micro-determination of cysteine, methionine and L- tryptophan, based on a Landolt-type clock system. The reaction has been followed potentiometricaly. The method is sensitive, simple and allows the determination of the aminoacid concentration in pharmaceutical products with very good accuracy. The proposed method was compared to other standard method or to the certified content given by manufacturers.

Numerous pharmaceutical products contain various aminoacids that are essential for protein synthesis in living cells. There are 22 essential aminoacids combining to give around 22^{500} protein molecules [1]. Therefore they are essential for the growth of organic tissues [2]. It is worth mentioning that all of them are of L configuration. The D configuration has been found in some plants and micro-organisms [3]. Part of them are not synthesized by human body and it is necessary to be contained in food or taken as adjuvants with some medication.

The specific methods of aminoacids determination are rarely used. When mixture of aminoacids are analysed, as a rule, they are combined with the chromatographic methods [4,5,6] either paper, thin layer, ionic exchanger, gas or liquid chromatography [7,8], in order to separate them.

The purpose of this work is to determine cysteine, methionine and L-tryptophan from pharmaceuticals by a kinetic clock reaction method, using a Landolt redox reaction system [9]. Cysteine (L-2-amino-3-thio-propionic acid) and methionine (2-amino-4-(methylthio)-butiric acid) are from the group of thioaminoacids, while L-tryptophan or L-α-amino-β-indol-3-il-propionic acid, a precursor of serotanin and melatonin is from the group of heterocyclic aminoacids [10]. The method consists of three redox reactions: first bromate-bromide generating bromine in a relative slow process, the second involving the analyte consuming bromine in a fast process and the third consisting of the oxidation of substrate (analyte) by bromate in very slow process. The method is quite cheap, relative rapid and makes use of simple and available techniques.

Experimental

Chemicals. Stock solutions of $KBrO_3$ $3 \cdot 10^{-2}$ M , KBr 1.0 M and $HClO_4$ 0.5 M were prepared in four-distilled water by weighting or being standardised by titrimetric methods. The amino acid solution was freshly prepared before each set of runs. All the substances were of analytical grade purity and used without further purification. Aminoacids were either analytical grade or for pharmaceutical purposes. In order to obtain calibration lines, each reaction mixture had a total volume of 25 ml. The order of adding reagent solutions into the reaction vessel was KBr, $HClO_4$, the aminoacid solution and four-distilled water up to 22.5 ml. After circulating water from a thermostat for at least 10 minutes, 2.5 ml $KBrO_3$ previously kept in a temperature bath were rapidly injected into the stirred mixture. This moment has been considered the zero time for the run.

Experimental Device. Measurements were carried out by using a Digitronix DXP-2040 (Seiko) potentiometer [11], a data acquisition device and a computer. The measuring electrode was a platinum plate. A saturated calomel electrode was the reference. The scheme for experimental set-up is given in Fig. 1.

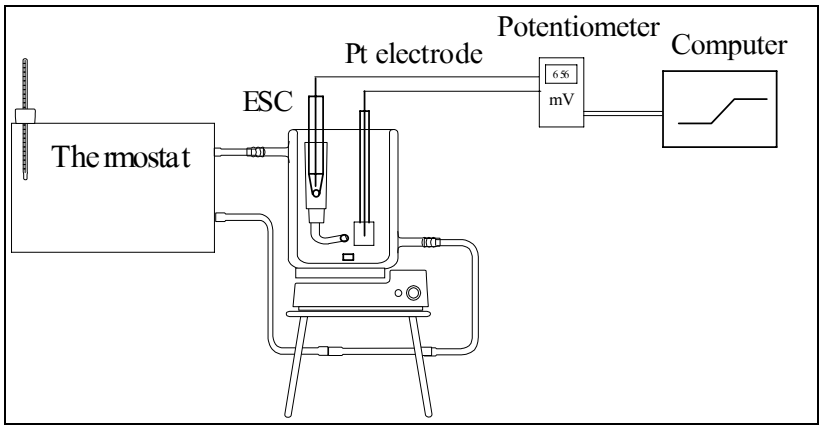

Fig.1. Schematic presentation of the experimental device.

Results and discussion

The successive and parallel processes consuming bromate (and bromine) for the simplest system under consideration, BrO_3^--Br^--cysteine are:

$BrO_3^- + 5Br^- + 6H^+ \rightarrow 3Br_2 + 3H_2O$	relative slow	(1)
$Br_2 + 2Cys \rightarrow 2Br^- + 2H^+ + (Cys)_2$	fast	(2)
$BrO_3^- + 6Cys \rightarrow Br^- + 3(Cys)_2 + 3H_2O$	slow	(3)

Here, Cys stands for cysteine ($HSCH_2CH(NH_2)COOH$ and $(Cys)_2$ stands for cystine, a disulphide ($HOOCCH(NH_2)CH_2)_2S_2$), the oxidation product of cysteine. The kinetics of the reaction (1) is known [12]. The oxidation of cysteine by bromine (2) is a rapid process. The oxidation of cysteine by bromate, under experimental conditions employed, is a slow process. A test reaction in the absence of bromide proved that. An unnoticed modification of electrode potential has been measured for a long time elapse. When bromide is present from the beginning, the process takes place more rapid. Therefore, the oxidation of cysteine proceeds mainly by means of bromine. The considered amino acids are traps for bromine. It means that bromine concentration reaches a low steady-state value, as long as cysteine exists in the mixture. When cysteine has been completely consumed, bromine accumulates in the mixture, and a modification of the potential of the redox electrode takes place. By choosing appropriate concentrations for BrO_3^-, Br^- and H^+, the reaction rate is maintained quite constant for small degree of bromate consumption. Bromide is restored by reaction (2), bromate amount reacted is small because of the stoichiometry. Hydrogen ion concentration should be maintained constant, either by using buffered solutions or a large excess. Therefore, within equal time intervals the same amount of bromine is generated, and consequently, the same amount of cysteine is oxidized. Hence

$$\text{rate} = \frac{-\,d[Cys]}{dt} = \frac{-\,\Delta[Cys]}{\Delta t} = \frac{[Cys]_t}{\tau} \qquad (4)$$

Under such circumstances, rate being constant, the concentration of cysteine in the mixture is proportional to the time elapse τ until the bromine concentration increases steeply.

Methionine behaves similarly, the oxidation yields a sulfoxide. The stoichiometry has been determined by a spectrophotometrical titration, bromine being generated from bromate in the presence of methionine under conditions employed in analytical measurements. The molar ratio was 1:1. Tryptophan reacts with bromine by a bromination process at the aromatic ring, consuming it rapidly. The molar ratio bromine:tryptophan was 2:1 indicating a double bromination.

The evolution of platinum electrode potential with time exhibits a shape of a titration curve, shown in Fig. 2. It has an inflexion point. The complete comsumption of the analyte corresponds to the moment of steep increase of the potential. The bromine evolution, as a result of reaction (1) only, is similar to the above described behaviour, but takes only a few seconds. The complete consumption time is in fact the diffference between the two inflexinon points, for the probe and the blank one. Nevertheless, we consider the end point of the reaction as being that of the inflexion, the blank probe needs no more than 2 - 3 seconds under the conditions employed. Since the same conditions are maintained for calibration line and measurements on real samples, no error is made by such an approach.

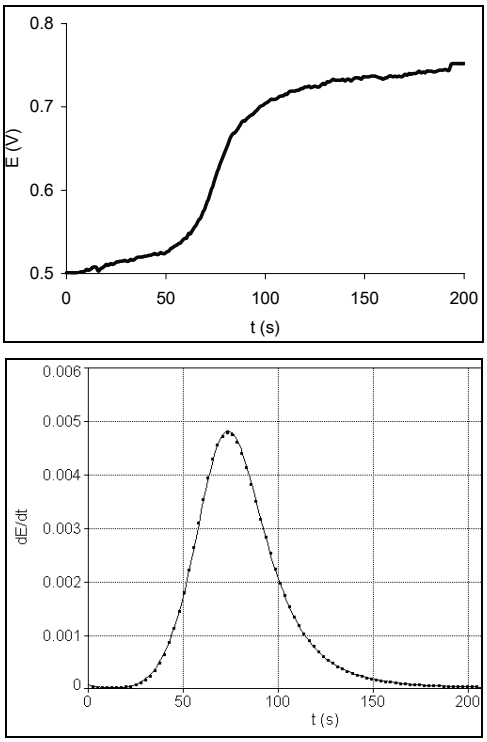

Fig. 2. Redox potential dependence on time and its derivative dE/dt = f(t) at [methionine] = $8 \cdot 10^{-5}$ M

The time for the inflexion can be determined more precisely from the derivative curve (also shown in Fig 2). This time period is proportional to the analyte concentration.

A plot of this time values, as a function of the aminoacid concentration is linear. Such a straight-line dependence represents the calibration line.

Searching for appropriate conditions.

a).The effect of the acidity. Several reaction time were determined using various concentration of mineral acid. The hyperbolic aspect of the curve confirms the second-order dependence of the rate with respect of hydrogen ion for the bromate-bromide reaction [9]. A value of mineral acid of 0.1 seems to be suitable for the analysis. It ensures a constant acidity within the initial period. Only minor hydrogen ion consumption occurs, although the reaction (1) stoichiometry requires 6 hydrogen ions.

b). The effect of potassium bromide. Eight bromide concentration values between 0.03 and 0.4 M were used to determine reaction time. A slope of 1.00 ± 0.07 from the graph lg $(1/\tau)$ - lg $[Br^-]$ confirming the first-order

dependence of the rate of bromate – bromide reaction [9]. A value of 0.2 M was chosen for the experiments to obtain calibration lines, ensuring its constant concentration.

c). Dependence on the conversion degree of bromate. By using different initial concentration of bromate and considering the degree of reaction defined by equation (5)

$$X = \frac{[BrO_3]_0 - [BrO_3]_{consumat}}{[BrO_3]_0} = \frac{\Delta[BrO_3]_0}{[BrO_3]_0} = \frac{[a\,min\,oacid]_0}{[BrO_3]_0} \qquad (5)$$

a linear dependence, as shown in Fig. 3, has been obtained, proving in fact the first- order dependence on bromate for the reaction (1) [9]

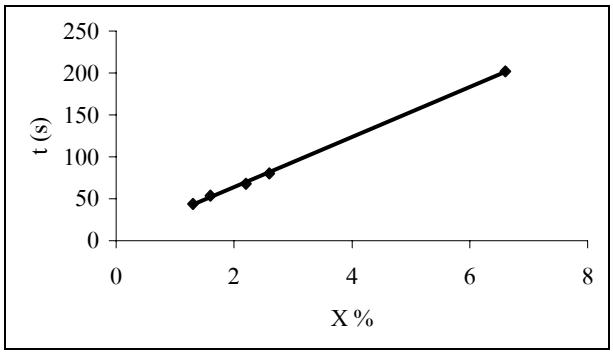

Fig.3. Dependence of reaction time on conversion using various initial concentration of bromate, [KBr] =0.2 M, [$HClO_4$] = 0.1 M, [tryptophan] = 4 x 10^{-5} M

A degree of reaction of 2.6 % has been chosen. It is in the range of initial rate determination ensuring a relatively constant concentration of bromate, and time values not too short to decrease the sensibility.

Calibration lines.

Under the same experimental conditions and using various known concentrations of the analytes, calibration lines were drowning. Figure 6 presents the data concerning tryptophan. The equation describing the line is:

$$t = (2{,}9 \pm 3{,}3) + (1{,}90 \pm 0{,}03) \cdot 10^6\,[tryptophan]_0 \qquad (6)$$

The correlation coefficient is r = 0.9995 and a standard deviation of 4.1 has been determined. The relative standard deviation (RSD) for a measurement is 2.2% for 5 individual measurements. Detection limit is 1.9x 10^{-6} M in accordance with IUPAC recommendation [14] It is obvious that the intercept is nearly zero as expected. The large slope indicates a good analytical sensitivity.

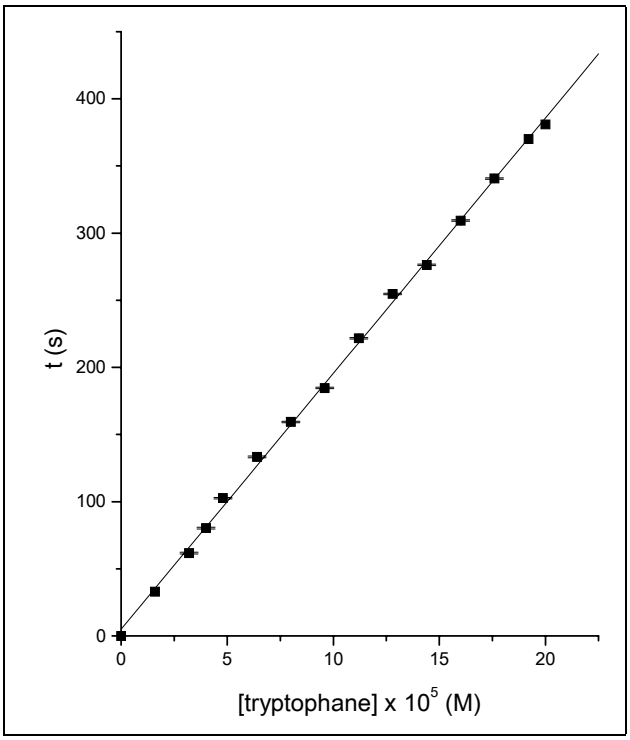

Fig. 4. Calibration line for tryptophan under the experimental conditions [KBr] = 0,2 M, $[KBrO_3] = 3 \cdot 10^{-3}$ M, $[H^+]$ = 0,1 M, and T = 293 K

The calibration line for methionine has been obtained by using the same redox system. The experimental conditions are [KBr] = 0.2 M, $[H^+]$ = 0.1 M, $[KBrO_3] = 3x10^{-3}$ M and T=293. A good straight line, with a very good correlation coefficient of 0.9991, has been obtained. It is described by:

$$t = (-6,0 \pm 4,2) + (1,56 \pm 0,06) \cdot 10^6 \left[\text{methionine}\right]_0 \quad (7)$$

The standard deviation for the line is 3.6 and a relative standard deviation of one point is 2.5 % using 5 individual measurements. The detection limit of $4.0x\ 10^{-6}$ M has been calculated. In this case also, the sensibility of the method is quite good.

The calibration line in the case of cysteine has been determined similarly, under the following conditions [KBr] = 0.2 M, $[H^+]$ = 0.1 M, $[KBrO_3]=3.0x10^{-3}$M and T = 293 K using 10 different concentrations of cysteine as trapping agent, each experiment having 3 - 7 replicate runs. The line is described by the equation

$$t = (-1,5 \pm 3,1) + (1,34 \pm 0,04) \cdot 10^6 \left[\text{Cys}\right]_0 \quad (8)$$

The standard deviation for the line is 2.7 and a relative standard deviation of one point is 2.8 % using 7 individual measurements. The detection limit is $1.7x10^{-6}$ M. It is worth mentioning here, that cysteine can be determined with another system of Landolt type reactions, using hydrogen peroxide - iodide reaction and cysteine being a trap for iodine [14]. Methionine and tryptophan do not react under such conditions, and cysteine concentration can be obtained from a mixture containing these aminoacids.

Figure 5 presents a calibration graph for methionine and its mixture with a constant concentration of cysteine. The effect of cysteine is additive because the calibration lines are parallel (the slopes almost the same).

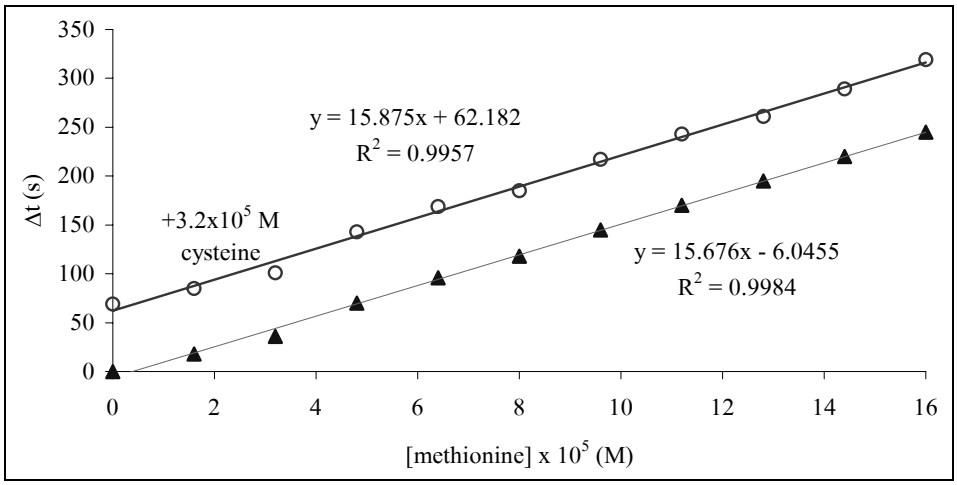

Fig. 5. Calibration line without and in presence of a constant concentration of cysteine 3.2 10^{-5} M.

The differences in the slopes of the three calibration lines are directly related to the reactivity of each aminoacid towards bromine, taking into account their structure and the reaction products. Nevertheless, the bromine consumption is quite rapid for all three analytes as compared to bromate oxidation. Consequently, the steady-state concentrations of bromine are slightly different for these reactions.

Several aminoacids, with possible interference were tested. As seen in Table 1, hystidine, tyrosine, guanine and phenylalanine do not interfere with the measurements, even in concentrations up to $1.0x10^{-3}$ M.

Table 1

Essential aminoacids that do not interfere in the determination of $4 \cdot 10^{-5}$ M methionine

Additive	Concentration ratio additive/ methionine)	Recovery %
Hystidine	100	102
Tyrozine	120	99
Guanine	150	99
Phenyl alanine	100	98

Validation on real samples

Methionine has been determined from the pharmaceuticals Mecopar forte, Metaspar and Infesol. The measurements were carried out by using the proposed clock method and the corresponding calibration lines as well as by using a spectrophotometrical method [15,16], The results were compared with certified content given by the manufacturers. For example, for the drug Mecopar forte the results are presented in Table 2.

Table 2

The content of methionine per tablet of Mecopar Forte

Methionine mg/ tablet	RSD %	Mean value mg
101	1	100
99		
100		
99		
101		

The amount of methionine contained in the product Metaspar, has been also obtained. The results are collected in Table 3. Five tablets were weighed, ground to powder, dissolved, filtered out and made up the solution to a volumetric flask. Various aliquots were measured and the content determined by means of the calibration line. The results were expressed as mg per tablet. The replicate runs gave reproductible results.

Table 3

Methionine Content of the pharmaceutical product Metaspar

Methionine found [mg]	RSD %	Mean content mg
65.6	0.2	65.64
65.7		
65.6		
65.8		
65.5		

Both series of measurements, the proposed one and the spectrophotometrical one, as an internal norm for its determination [15,16]. gave similar values with the manufacturers' certified contents [17] (see also Table 4)

Another determination of methionine was made from the complex product Infesol 40, manufactured by *Berlin-Chemie Menarini Group*.A. It is a solution containing many aminoacids [17]. A sample of 0.125 ml of solution was introduced in the reaction mixture and the consumption time determined. No separation or filtration was necessary. No one of the aminoacids contained in the solution interferes. The comparative results are given in Table 4. Recovery (R %) is between 99 and 100.6 %

Table 4

Methionine content in Mecopar forte, Metaspar and Infesol. 40

Pharmaceutical form		Landolt method		Spectrophoto-metric method		Manufacturers' certified content	R %
		mg*	RSD	mg*	RSD	mg*	
Metaspar	Capsule	64	0.8	65.6	0.2	64.5	99
Mecopar Forte	Tablet	100.5	0.5	100	1	100	100.5
Infesol 40	Perfusable solution	1760	0.2	-	-	1750	100.6

*) the content refers to mg/capsule, mg/tablet, and mg/100 ml solution respectively.

As seen in the table 4, the results are similar to those obtained by spectrophotometrical method and the certified content given by producers for all investigated pharmaceuticals. The data show the reliability of the proposed kinetic method for the determination of methionine.

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